Follicular lymphoma-like B cells of uncertain significance (*in situ* follicular lymphoma) may infrequently progress, but precedes follicular lymphoma, is associated with other overt lymphomas and mimics follicular lymphoma in flow cytometric studies

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ABSTRACT

In situ follicular lymphoma, more recently known as follicular lymphoma-like B cells of uncertain/undetermined significance is well accepted. However, the morphological criteria have evolved since it was first described and data are limited and conflicting regarding its clinical implications and whether the extent of involvement predicts an association with overt lymphoma. It is also unknown how often it will be identified by flow cytometric studies and how often it precedes overt follicular lymphomas. A multiparameter study of 31 biopsies with follicular lymphoma-like B cells of uncertain significance and 4 'benign' lymph node biopsies that preceded an overt follicular lymphoma was, therefore, performed. Fifty-two percent of biopsies with follicular lymphoma-like B cells were associated with a prior or concurrent lymphoma but only 6% subsequently developed lymphoma (median follow up 26 months). Neither the number, proportion or density of BCL2$^+$ germinal centers were associated with overt follicular lymphoma/diffuse large B-cell lymphoma. Flow cytometric studies identified follicular lymphoma-like B cells in 8 of 15 evaluable cases. The proportion but not the absolute number of BCL2$^+$ germinal centers was associated with the likelihood of positive flow cytometric studies (P<0.01). All 4 'benign' biopsies that preceded an overt follicular lymphoma demonstrated follicular lymphoma-like B cells. Thus, although few patients with follicular lymphoma-like B cells of uncertain significance progress within the follow-up period, it at least precedes many follicular lymphomas. The extent of involvement does not predict the occurrence of prior or concurrent lymphomas. Flow cytometric studies demonstrating follicular lymphoma-like B cells must not be over-interpreted as they may only reflect follicular lymphoma-like B cells.

Introduction

In situ follicular lymphoma (FL), initially reported in 2002, was defined as lymph nodes (or other lymphoid proliferations) with an intact architecture but with scattered germinal centers (GC) with variably dense populations of BCL2$^+$ CD10$^+$ lymphoid cells that are usually predominantly centrocytes, are monoclonal, and have a t(14;18) *IGH/BCL2* translocation.[1] While the individual abnormal follicles are indistinguishable from those in FL, it is considered important to recognize these cases because a significant proportion of the patients do not have concurrent or subsequent overt FL. *In situ* FL was recognized as an FL variant in the 2008 WHO classification, and because of concern that they were not an overt lymphoma, the name "intrafollicular neoplasia" was introduced as optional diagnostic terminology. More recently, the name "follicular lymphoma-like B cells of uncertain or undetermined significance" (FLBUS) was suggested to emphasize that these cases are not considered overt malignant lymphomas.[2,3]

A number of important uncertainties still remain, in part because of the very few published studies with inconsistent results and, in part, because the criteria for recognizing these cases have evolved. The initial report suggested that 44% of patients would have an associated FL, including 23% who developed an FL, and a small study from another group reported an associated FL or diffuse large B-cell lymphoma in 38%, including the 2 of 13 (15%) patients with subsequent FL or DLBCL.[1,4] The latter series also reported only 38% of cases with no lymphoma at all, as several patients had lymphomas not of FL/DLBCL type.[4] Subsequently, a report from the initial group[5] distinguished *in situ* FL from "partial nodal involvement by FL" (PFL), re-categorized some of their original cases and added new ones, and then found that 18% of the patients had a prior or concurrent FL and only 5% developed a subsequent FL. Some patients also had other types of B-cell lymphoma. An even more recent small study by other investigators found synchronous (n=6) or subsequent (n=2) B-cell lymphomas in 61% of cases with *in situ* FL.[6] In addition to uncertainties concerning the incidence of associated overt lymphomas, whether the extent of FLBUS can be used to predict which cases will be associated with an overt lymphoma is also controversial.[4,5] Nothing is known as to how often flow cytometric immunophenotypic studies (FCS) will detect FLBUS. This is an important question given the heavy reliance on cytopathological diagnostic procedures to evalu-

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ate lymphadenopathy, since the presence of a population of cells with an FL-like phenotype is often used to support the diagnosis of FL or one of the other GC-associated lymphoid neoplasms. Finally, because the proportion of overt FL preceded by FLBUS is not known, it remains to be established whether the situation is analogous to that of chronic lymphocytic leukemia where virtually all cases are preceded by monoclonal B-cell lymphocytosis.[7]

To address these questions, 31 cases of FLBUS were investigated, including quantitative assessment of the FL-like follicles and the results correlated with the clinical findings. In addition, FCS had been performed in 17 cases. Four cases of overt FL were also identified in which a prior 'benign' lymph node biopsy could be obtained and stained for FLBUS.

Methods

Case selection, pathological and clinical review

The study was performed in accordance with the guidelines of the Institutional Review Board of the University of Pittsburgh School of Medicine. Thirty-one specimens, including 28 lymph nodes and 3 extranodal tissues (tonsil, adenoids, colon) that fulfilled the criteria of Jegalian *et al.*[5] for *in situ* FL, were selected from the files of the Division of Hematopathology of the University of Pittsburgh Medical Center Presbyterian Hospital. Cases of partial involvement by FL were excluded. Routine histological sections were reviewed and the following were recorded: GC size, GC borders, predominance of centrocytes and attenuation of mantle zones. The approximate area of each tissue section was calculated based on the largest perpendicular diameters. Any concurrent pathological findings were also recorded. Clinical data including age, gender, concurrent, prior or subsequent malignant lymphoma, stage at diagnosis, type of therapy and follow-up data from date of first diagnosis of FLBUS were also reviewed.

Immunohistochemical and flow cytometric immunophenotypic studies

All available paraffin section immunohistochemical stains were reviewed, and when not available, the following stains were performed on formalin-fixed paraffin embedded sections using an automated immunostainer with CC1 antigen retrieval (BenchMark, Ventana, Tucson, AZ, USA): CD3 (polyclonal, dilution 1:100; DAKO, Carpinteria, CA, USA), CD5 (SP19, pre-diluted, Ventana), CD20 (L26, dilution 1:50; DAKO, Carpinteria, CA, USA), CD10 (56C6, pre-diluted, Ventana), BCL6 (PG-B6p, dilution 1:5; DAKO) and BCL2 (124, pre-diluted, Ventana). Ki-67 immunohistochemical stains were performed manually using the MIB-1 clone (Dako Corporation; Carpinteria, CA, USA). The following parameters were recorded for the BCL2 stained sections: total number of GC present, number of $BCL2^+$ positive GC, extent of involvement by the neoplastic cells in each $BCL2^+$ GC estimated as less than 25%, 25-50%, 50-95% or more than 95%, and relative intensity of BCL2 staining in positive cells in the GC.

The results of FCS, which had been performed using 4 or 8 color analyses in 17 cases with a panel of antibodies to detect at least CD19, CD20, CD10, Kappa and Lambda were reviewed (FACS Canto flow cytometer, data analyzed using Cell Quest or FACS Diva software, Becton Dickinson, San Jose, CA, USA). In one of the cases, histograms from studies performed at another institution were reviewed. FCS for BCL2 was performed and interpreted as previously described[8] in 12 of 17 cases. Subsequently, 2 cases with $CD10^+$ $BCL2^+$ light chain class restricted B cells were excluded since there was a concurrent $CD10^+$ B-cell lymphoma in the tissue submitted for flow cytometry.

Cytogenetic FISH studies

Cytogenetic FISH studies were performed on paraffin sections in the 3 concurrent and one subsequent DLBCL, as previously described[9] using the following probes: Vysis LSI *BCL6* dual-color break apart and Vysis LSI *BCL2* dual-color break apart (Abbot Molecular, Des Plaines, IL, USA).

Retrospective analysis of 'benign' lymphoid tissue in patients with a subsequent overt FL

Lymph node biopsies that had been considered 'benign' were identified in 4 patients with a subsequent overt FL. The histological sections were reviewed and, unless already available, CD10, BCL2, CD3 and CD20 immunohistochemical stains were performed and reviewed. One other case was reviewed but excluded as a remote history of prior FL was mentioned although there was no documentation of it in our database.

Statistical analysis and imaging

Numerical variables were compared with the Mann Whitney U test using GraphPad Prism 5 software (Graph Pad Software, La Jolla, CA, USA). Photomicrographs were acquired with an Olympus DP71 camera and software (Olympus America Inc.) mounted on an Olympus BX51 microscope. Images were finalized with Adobe Photoshop CS4 (extended version).

Results

Clinical features and association with other lymphomas

Most cases occurred in older adults (range 38-88 years, median 72 years) with a male:female ratio of 2.1:1 (Table 1). Three of 31 (10%) FLBUS cases were associated with a history of prior FL, including one patient who had concurrent classical Hodgkin lymphoma and one who had a concurrent peripheral T-cell lymphoma and a subsequent classical Hodgkin lymphoma, and one (3%) with prior chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL). Twelve of 31 cases (39%) were associated with a concurrent malignant lymphoma, including 4 FL (13%), 3 DLBCL (10%), one CLL/SLL, one extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue type, one *in situ* mantle cell lymphoma/mantle cell lymphoma-like B cells of uncertain significance (MCLBUS), one peripheral T-cell lymphoma and one mycosis fungoides. One patient developed gastric diffuse large B-cell lymphoma one month after the diagnosis of FLBUS. Fourteen patients (45%) had no known prior, concurrent or subsequent lymphoma. One of these patients did have 2 biopsies eight months apart demonstrating FLBUS. Median follow up for the entire group ranged from 0-71 months (median 26 months).

Of the 14 patients who had no associated malignant lymphoma, one was treated with R-CVP (rituximab + cyclophosphamide, vincristine and prednisone), one with radiotherapy, 8 were observed and no treatment data were available in 4. After a median follow up of 30 months (range 1-68 months), 9 had no evidence of progression, one patient died of unknown causes, and 4 were alive with no available follow up. Of the 9 patients with a prior or concurrent FL or DLBCL with available follow up, the 3 patients with DLBCL were treated with R-CHOP (rituximab + cyclophosphamide, doxorubicin hydrochloride, vincristine and prednisone) for their DLBCL, 5 were observed, and treatment data were unknown in one. All were alive after a median follow up of 11 months (range 3-71 months). In addition, there were 6 patients with prior

Table 1. Clinicopathological features of FLBUS cases.

Case ID	Sex	Age	FLBUS Bx	FLBUS Other Inv	Other pathology/ Site	Interval (mo)[a]	Stage	Rx	FU (mo)[b]	Status at FU
1	M	48	R adenoids				NA	OBS	10	A without D
2	F	66	L cervical LN				NA	OBS	11	A without D
3	F	72	mesenteric LN	bilateral hilar LN (CT)	FL 1-2, mesenteric LN	0	IV	OBS	9	AWD (adenopathy)
4	M	88	R cervical LN		CLL/SLL, R cervical LN	0	NA	NA	66	Dead
5	M	75	mediastinal LN		MZL (MALT), lung	0	NA	NA	39	A without D
6	F	88	R axillary LN	L axillary LN (CT)	PTCL, R axillary LN	0	NA	NA	23	Alive
7	M	47	R inguinal LN				NA	NA	68	Alive
8	F	60	ileocolic LN				NA	OBS	21	A without D
9	M	74	R level 4 LN		Non-necrotizing granuloma, R Level 4 LN	0	NA	OBS	38	A without D
10	M	65	L axillary LN	retroperitoneal LN (CT)			IIIA	R-CVP x 6	53	A without D
11	M	76	R axillary LN (BX) R inguinal (CT), L axillary (CT)	L inguinal LN	MF,	0	III	ECP, XRT	26	AWD (MF)
12	M	71	mesenteric LN		DLBCL, colon	0	IV	R-CHOP x 6	34	A without D
13	M	85	pancreatic LN		IgG4+ plasma cells	0	NA	NA	1	Dead
14	F	82	cystic LN		FL1-2, gall bladder	0	NA	NA	11	Alive
15	F	55	L axillary LN	L cervical LN (BX)			NA	NA	58	Alive
16	M	77	R cervical LN	left post triangle, left hilar, portocaval, inguinal LNs (CT)	FL, orbit	-8	NA	OBS		
					CHL, R cervical LN	0	NA	ABVD + Rituxan	42	A without D
17	M	72	R deep cervical		FL, colon polyp PTCL, R deep cervical LN CHL	-3 0 63	NA IIIE IIIA	OBS R-CHOP x 6 ABVD x 4	71	AWD (adenopathy)
18	M	78	L inguinal LN		*In situ* MCL, L inguinal LN	0	NA	NA	35	A without D
19	F	85	L inguinal LN		CLL/SLL, PB	-61	NA	NA	2	DWD (CLL)
20	M	86	L periaortic LN				NA	NA	28	A without D
21	M	40	R inguinal LN				NA	XRT	31	A without D
22	M	86	inguinal LN				NA	OBS	55	Alive
23	F	82	R inguinal LN	Retroperitoneal	DLBCL, stomach	1	NA	NA	1	DWD (DLBCL)
24	M	79	L axillary LN	LN (CT)			NA	OBS	45	Alive
25	F	63	L cervical LN		DLBCL, L cervical LN	0	IA	R-CHOP x 3, XRT	39	A without D
26	M	69	mesenteric LN		FL1-2, small bowel	0	NA	OBS	3	A without D
27	M	50	R axillary LN		Increased IgG levels		NA	OBS	9	A without D
28	M	61	colon		DLBCL, R colic LN	0	IAE	R-CHOP x 3	6	A without D
29	M	65	R tonsil		FL1-2, L tonsil FL 3A & DLBCL, spleen	0 5	NA NA	OBS Splenectomy, OBS	5	A without D
30	F	38	R inguinal LN		FL1-2, L antecubital LN	-8	NA	NA	0	AWD (adenopathy)
31	M	67	B/L axillary LN		splenomegaly	0	NA	OBS	10	A without D

CT; computed tomography scan; BX: biopsy proven; CLL/SLL: chronic lymphocytic leukemia/small lymphocytic lymphoma; MZL (MALT): marginal zone lymphoma of mucosa associated lymphoid tissue type; PTCL: peripheral T-cell lymphoma; CTCL: cutaneous T-cell lymphoma; MF: mycosis fungoides; DLBCL: diffuse large B-cell lymphoma; FL: follicular lymphoma; MCL: mantle cell lymphoma; R-CVP: rituxan, cyclophosphamide, vincristine and prednisolone; ECP: extra-corporeal photopheresis; ABVD: doxorubicin, bleomycin, vinblastine, dacarbazine; R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone; A without D: alive without disease; AWD: alive with disease (secondary lymphoma); DWD: dead with disease; D without D: dead without disease; alive: alive, disease status unknown; dead: dead, disease status unknown; [a]Interval from FLBUS to or from other pathology, as indicated in preceding column. [b]Follow-up duration was determined from the time of diagnosis of FLBUS.

or concurrent non-GC derived B-cell or T-cell lymphomas, 2 of whom died (#4 and #19, both with concurrent CLL/SLL) (median follow up 31 months, range 2-66 months). The single patient who developed gastric DLBCL died within a month after the FLBUS diagnosis shortly after the DLBCL was diagnosed.

Histopathological/immunophenotypic features of FLBUS and correlation with clinical findings

Except in cases with concurrent involvement by a secondary lymphoma, all cases showed architectural preservation with a median of 34 (range 3-916) usually small to medium sized GC in the tissue examined (Figure 1). The median density of GC was 49/cm^2 (range 2-331). Mantle zones were unremarkable in 22 of 31 cases but were expanded in 8 and attenuated in one. BCL2$^+$ cells in the germinal centers in all cases showed greater intensity of staining compared to the mantle zones (Figure 1). In 2 of 31 cases, the intensity of the BCL2$^+$ cells in some GC was decreased compared to other BCL2$^+$ GC. BCL2$^+$ GC areas in all cases were composed almost exclusively of centrocytes.

The biopsies with FLBUS demonstrated from 1 to 207 BCL2$^+$ GC (median 10) with no significant differences between the 11 cases with (median 17, range 2-115) and the 20 cases without (median 9, range 1-207) an associated FL or DLBCL (Table 2, Figure 2A). In addition, neither the proportion (median 67% *vs.* 68%) nor the density (21 follicles/cm^2 for both groups) of BCL2$^+$ GC showed significant differences between these 2 groups (Table 2, Figure 2B). Significant differences were also not found between the 2 groups based on the extent of involvement of individual follicles (*not all data shown*, Table 2, Figure 3) or the density of BCL2 positive follicles (Figure 4). Further subset analysis of these parameters for the FL/DLBCL cases with a history of prior FL compared to those cases with a concurrent FL or DLBCL also showed no significant difference. Similar results were obtained when the FL and DLBCL cases were compared separately with the 20 cases without an associated FL or DLBCL.

Flow cytometric immunophenotypic studies

Flow cytometric immunophenotypic studies (FCS) showed CD10$^+$ light chain class restricted (LCCR) B cells in 7 of 15 tested cases with FLBUS (Table 3, Figure 5). In addition, one case had demonstrable CD10$^+$ BCL2$^+$ cells that were surface immunoglobulin negative. The FL-like B cells (CD10$^+$ B cells with either LCCR and/or BCL2$^+$) comprised 1-12% of total events (median 3, mean 4.6±4.0) and 2-35% of B cells (median 14.6, mean 16.1±12.6). The cases with demonstrable FL-like B cells had a significantly increased proportion of BCL2$^+$ follicles compared to those without the FL-like B cells (median 91%, range 65%-100% *vs.* 42%, 0.3-91%; P<0.01). However, there was no significant difference in absolute number of BCL2$^+$ follicles between the 2 groups (median 34, range 5-207 *vs.* 8, 3-56).

Cytogenetic FISH studies

Cytogenetic FISH studies for *BCL2* and *BCL6* translocations were performed in the 4 available biopsies with concurrent (n=3) or subsequent (n=1) DLBCL (Table 4). Only one case showed a possible *BCL2* translocation.

Retrospective analysis of prior 'benign' lymph node biopsies

Four 'benign' LN biopsies were identified that had been performed 1-108 months (median 27 months) prior to the diagnosis of an overt FL. The clinicopathological features are described in Table 5. All cases showed an intact architecture. BCL2$^+$ CD10$^+$ GCs were detected in all cases. The LN in the positive cases showed prominent GC in 2, and only occasional GC in the other 2 (Figure 6). Although not evaluable in one case because of limited tissue on the BCL2 stain, the absolute number of BCL2$^+$ GC was low and they were variable in size.

Flow cytometric immunophenotypic studies had been performed in only one case. They demonstrated approximately 1% CD10$^+$, CD20$^+$, BCL2$^+$, kappa restricted B cells out of a total of about 45% otherwise polytypic-appearing B cells with a κ/λ ratio of approximatelty 1.0 (some non-specific staining complicated interpretation).

Discussion

In situ localization of FL, soon after shortened to *in situ* FL, was initially described in 2002 as the presence of focal germinal centers (GC) with CD10$^+$, strongly BCL2$^+$ mono-

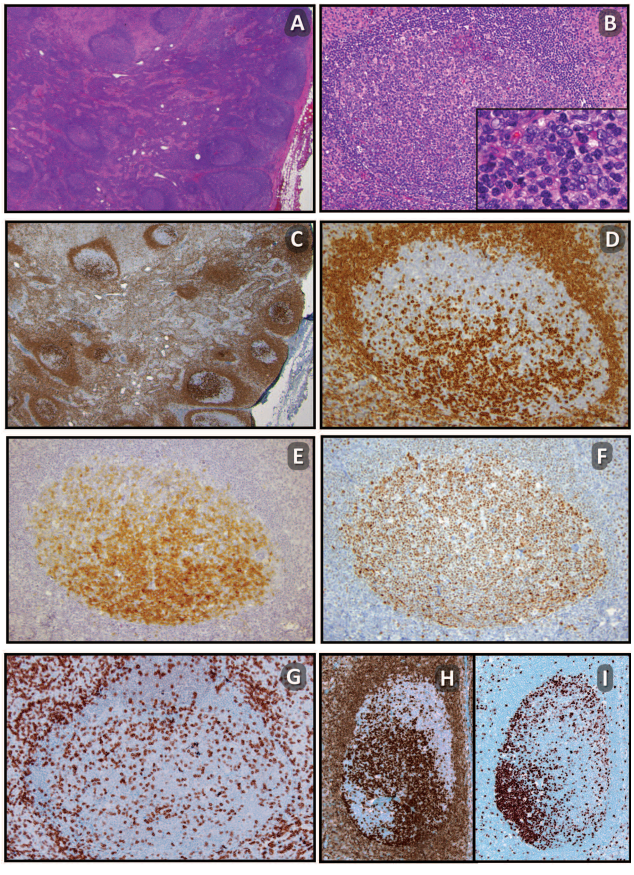

Figure 1. Lymph node with FLBUS (case 11). (A) Note the architectural preservation with scattered follicles. (B) Some GC have an increased proportion of centrocytes. (C) Many GC had variable numbers of BCL2$^+$ cells that were more strongly stained than the mantle zone B cells. (D) This GC shows strong BCL2 expression in the lower half (E) where the CD10 is also more strongly expressed. (F) In contrast, the BCL6 immmunostain shows similar positivity in both areas. (G) A CD3 stain shows scattered cells, more in areas where there are fewer CD10$^+$ BCL2$^+$ cells. (I) A Ki-67 stain of a different follicle seen in the lower right hand corner in C (rotated for this image) demonstrates a low proliferative index in the area with the (H) dense population of BCL2$^+$ cells. Original magnification x40 (A, C), x200 (H, I) and x400 (B, D, E–G).

clonal centrocytes in lymph nodes that otherwise had an intact architecture and follicular hyperplasia.[1] These authors emphasized how almost half of the cases represented early involvement of reactive GCs by FL, how the other cases might either be very early FL or a pre-neoplastic event, and, finally, how immunohistochemistry was useful for 'early diagnosis'. Surprisingly, there have only been a limited number of studies published over the subsequent decade, in spite of the fact that these cases were included as a variant of FL in the 2008 WHO classification of lymphomas as "*in situ* FL/intrafollicular neoplasia".[10] Subsequently, in order to emphasize the fact that these are not considered to be overt lymphomas, the name of follicular lymphoma-like B cells of uncertain or undetermined significance (FLBUS) has been suggested.[2,3] Consistent with the interest in not over-diagnosing lymphomas leading to patient anxiety and potential overtreatment, the reported apparent incidence of FLBUS is much greater than the incidence of FL (2.3% *vs.* approx. 0.03%).[11,12]

In 2011, the original group of authors published a second overlapping series of *in situ* FL where they refined their criteria and introduced the distinction from partial involvement by FL. Partial involvement of FL (PFL) is distinguished from FLBUS, because there is typically partial architectural alteration with clustering of the abnormal and often larger follicles that may have more centroblasts

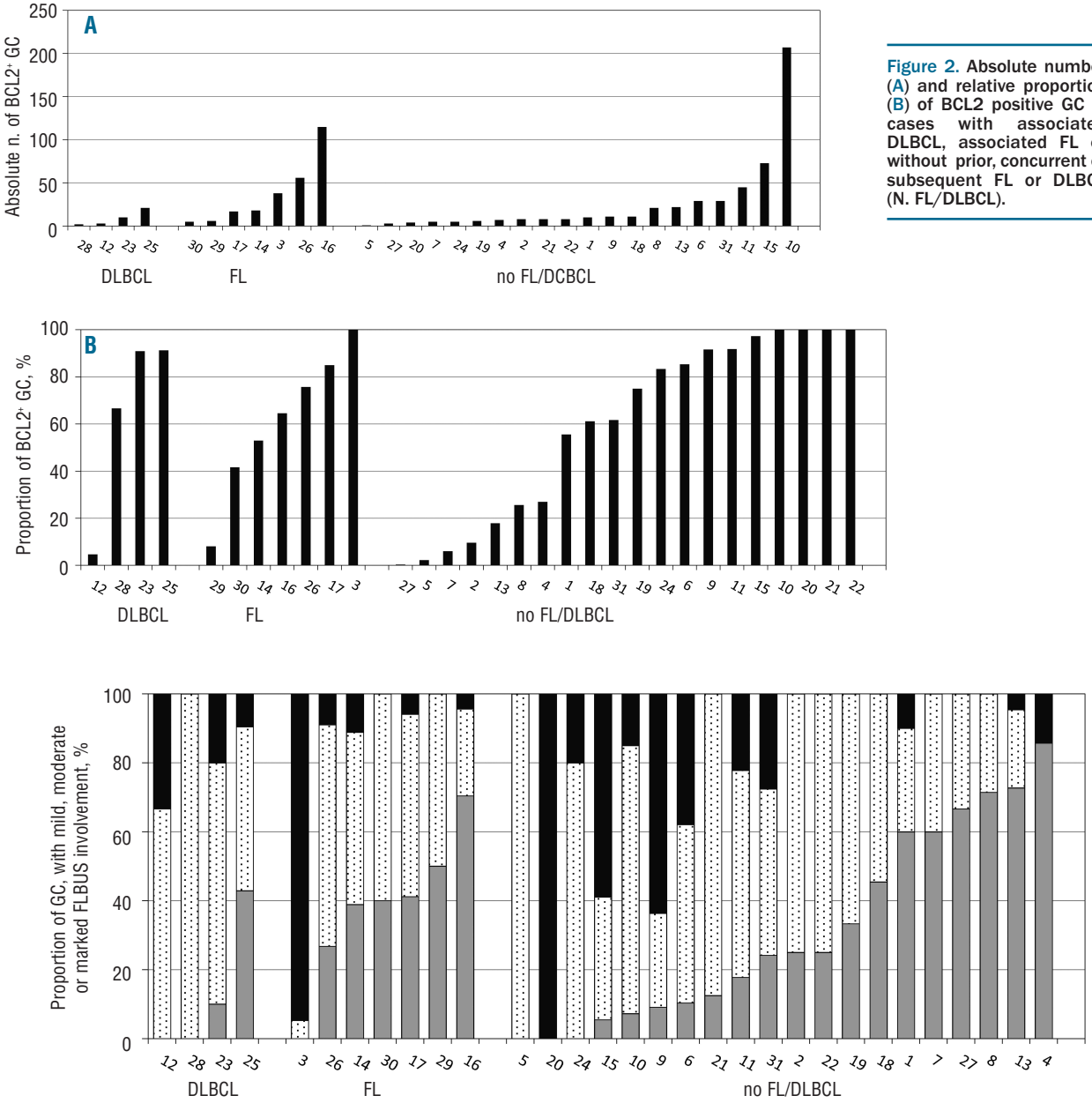

Figure 2. Absolute number (A) and relative proportion (B) of BCL2 positive GC in cases with associated DLBCL, associated FL or without prior, concurrent or subsequent FL or DLBCL (N. FL/DLBCL).

Figure 3. Proportion of GC with mild, moderate or marked FLBUS involvement in cases with associated DLBCL, associated FL or without prior, concurrent or subsequent FL or DLBCL (N. FL/DLBCL) - GC with <25% BCL2+ cells (solid gray), GC with 25-95% BCL2+ cells (dotted) and GC with >95% BCL2+ cells (solid black).

Table 2. Pathological findings correlated with presence or absence of other lymphomas, median (range).

	All FLBUS cases	Prior FL[a]	Concurrent FL/DLBCL[b]	Subsequent FL/DLBCL	Prior/concurrent other B-cell lymphoma[c]	Concurrent T-cell lymphoma	No lymphoma
N. of cases	31	3	7	1	4	2	14
Abs. n. of BCL2$^+$ GC	10 (1-207)	17 (5-115)	18 (2-56)	10	7 (1-11)	37 (29-45)	9 (3-207)
Abs. n. of BCL2$^+$ GC/cm^2	21 (1-146)	12 (7-70)	21 (2-146)	36	11 (1-64)	34 (31-37)	21 (1-113)
% GC BCL2$^+$	67 (0.3-100)	62 (42-85)	67 (5-100)	91	44 (2-75)	89 (85-92)	73 (0.3-100)
% of GC with >25% BCL2$^+$ cells	75 (14-100)	59 (30-60)	73 (50-100)	90	61 (14-100)	86 (82-90)	75 (27-100)
% of GC with >50% BCL2$^+$ cells	45 (0-100)	53 (21-60)	50 (33-100)	70	15 (0-45)	68 (60-76)	41 (5-100)
% of GC with >95% BCL2$^+$ cells	9 (0-100)	4 (0-6)	10 (0-95)	20	0 (0-14)	30 (22-38)	7 (0-100)

FL: follicular Lymphoma; FLBUS: FL-like B cells of uncertain significance; DLBCL: diffuse large B-cell lymphoma. [a]1 case had concurrent peripheral T-cell lymphoma and subsequent classical Hodgkin lymphoma and another case had concurrent classical Hodgkin lymphoma (not included in other columns). [b]One case had a concurrent grade 1-2 FL and developed a subsequent follicular lymphoma grade 3A of 3 FL with DLBCL (not included in other columns). [c]One case had a concurrent in situ mantle cell lymphoma which, similar to FLBUS, is of uncertain significance.

than seen in FLBUS, a less clear demarcation between the GC and more attenuated mantle zones, more variably intense BCL2 and CD10 staining by immunohistochemistry and sometimes extrafollicular CD10$^+$ BCL2$^+$ neoplastic cells. The cases in this series fulfilled the stricter FLBUS criteria. Sometimes, even the CD10 immunohistochemical stain is a clue to the abnormal follicles as the staining may be more intense than in the hyperplastic GC, just as FL tend to have stronger CD10 expression than hyperplastic follicles.[13] In all cases, the abnormal GC contained mostly centrocytes, similar to other reports in the literature[1,4,5] (although one abstract described FLBUS with predominantly centroblasts with FL grade 3 morphology) that are mostly BCL2 negative.[14]

Excluding PFL from *in situ* FL led to a fall in the proportion of patients developing subsequent FL from 23% to 5%, although another 18% of the patients did have a prior or concurrent FL.[5] Another study of only 13 cases of FLBUS reported the presence of an associated FL or DLBCL in 38% of patients including the 15% of patients who developed a subsequent FL or DLBCL.[4] The most recent study of 13 patients with *in situ* FL, reported synchronous (n=6) or subsequent (n=2) B-cell lymphomas in 61% of cases.[6] Given this high proportion of cases associated with other mostly clonally related B-cell lymphomas, these authors emphasized how "*in situ* follicular lymphoma" "might represent a phenomenon related to follicular homing of lymphoma, rather than being an attribute of pre-neoplastic FL precursors".[6] Although in most other studies the clonal relationship between the FLBUS and the associated overt lymphomas is unknown, there are other rare reports that they can represent the same clone, including one demonstrating secondary chromosomal abnormalities restricted to the overt lymphoma.[15,16] It should be recognized that, particularly when FLBUS is found in a patient with a prior documented FL, whether one has discovered a new or pre-existing pre-neoplastic population or subtle involvement by an overt lymphoma can not be determined at the present time. Even clonal identity does not answer this question. Whether the presence of additional molecular/cytogenetic abnormalities could provide an answer still remains to be determined. Studies assessing the relationship of FLBUS to associated overt B-cell lymphomas are difficult because of the paucity and focality of the abnormal cells in FLBUS and possibly biased to cases with more extensive involvement.

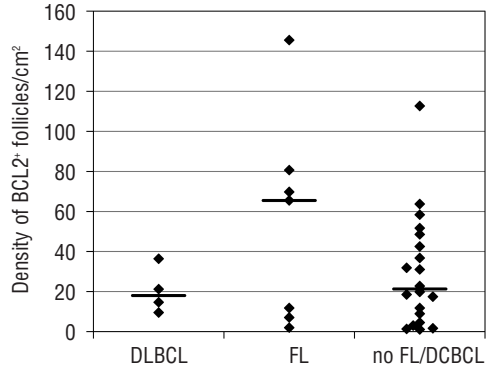

Figure 4. Density of BCL2$^+$ follicles in cases with associated DLBCL, associated FL or without prior, concurrent or subsequent FL or DLBCL (N. FL/DLBCL). The median for each group is also shown.

Consistent with the concept that FLBUS shows a slow rate of progression, only one patient in this series without an associated prior or concurrent FL or DLBCL developed a subsequent FL or DLBCL. It should be acknowledged, however, that the follow-up period in this and other studies remains relatively short and that the ultimate proportion of patients who will progress still has to be determined. On the other hand, more than half of the patients in this series and in two others had either synchronous or metachronous overt lymphomas of some type.[4,6] Because many patients with FLBUS do have prior or concurrent FL or other types of lymphoma, as also emphasized by others,[4,6] staging is important. However, with the diagnosis of FLBUS and no evidence of overt lymphoma elsewhere, our findings further support the recommended concept of

Table 3. Flow cytometric results in cases with FL-like B-cell population identified in cases without confounding concurrent FL/DLBCL.

Case ID	Basis for positivity	B cells[a]	Overall k:l ratio	CD10+ CD20+	CD10+ CD20+ Kappa+	CD10+ CD20+ Lambda+	CD10+ CD20+ BCL2+	CD10+ CD20+ BCL2 indet/neg	CD3%
3	CD10+ LCCR and BCL2+	35%	3.4	13%	12%	0.20%	12%	1%	54%
6	CD10+ LCCR and BCL2+	52%	1.1	3%	0%	3%	1%	3%	43%
10	CD10+ LCCR and BCL2+	48%	1.4	14%	8%	1%	14%	1%	44%
11	CD10+ LCCR and BCL2+	48%	1.0	5%	1%	3%[b]	2%	3%	44%
16	CD10+ SIg- and BCL2+	57%	2.0	20%	11%	6%	1%	20%	30%
21	CD10+ LCCR and BCL2 ND	20%	0.31	7%	0.50%	7%	ND	ND	54%[c]
23	CD10+ LCCR and BCL2 ND	6%[d]	0.57	2%	0.10%	1%	ND	ND	41%
24	CD10+ LCCR and BCL2+	16%	0.56	3%	1%	2%	2%	1%	81%

Because not all antibodies are run in the same tubes, numbers are not all comparable to one another. [a]Based on CD19 and/or CD20 [b]There are 2.3% lambda restricted CD10+ BCL2+ B cells with slightly dimmer CD20 compared to the other CD10+ B-cells that are BCL2-, have dimmer CD10 and brighter CD20. [c]Based on CD5, there was no CD3 stain. [d]Based on CD20. There were more numerous CD19+ cells related to technical aspects.

Table 4. Cytogenetic FISH results in DLBCL cases associated with FLBUS[a].

Case ID	Cytogenetic FISH result
12	nuc ish(5'BCL2x2~4,3'BCL2x2~5)(5'BCL2 con3'BCL2x1~4)[89/147][b], nuc ish(BCL6x3~4)[91/296]
23	nuc ish(BCL2x2), nuc ish(BCL6x2)
25	nuc ish(BCL2x1~2, nuc ish(BCL6x2~3)[c]
28	nuc ish(BCL2x3~5)[115/215]53.5%, nuc ish(BCL6x3)[28/246]11.4%

[a]Cytogenetic FISH studies were performed only on the DLBCL. [b]FISH was positive for 1 or 2 extra 3' proximal signals for BCL2 in 70/147 interphase cells examined, including 39 cells with 1 or 2 extra stable signals for BCL2. The remaining 19 abnormal cells had 1 or 2 extra stable fusion signals. [c]64/252 cells had only one signal for BCL2 (25.4%) and 127/289 had an extra signal for BCL6 (43.9%).

conservative management with routine follow up.[17] It is important to also recognize, however, that disseminated FLBUS is not equivalent to the presence of an overt lymphoma. One of the patients reported here had recurrent FLBUS; FLBUS has been reported to be present at two separate anatomic sites[18,19] and nodal FLBUS with clonally identical cells in the peripheral blood has been described.[20] This concept is also consistent with the observation that up to 66% of healthy individuals over the age of ten years have circulating cells with *BCL2* translocations, and with the belief that these cells already have many FL-like features and colonize the GC niche.[21-27]

Other non-FL/DLBCL B-cell, T-cell and classical Hodgkin type lymphomas have also been reported in 15-31% of patients with FLBUS. However, whether these bear any relationship to the FLBUS, reflect an increased propensity to develop lymphoma due to genetic or environmental factors, or simply result from the overt neoplasm leading to a tissue biopsy are all unknown.[4,5] Here there were 4 non-FL/DLBCL B-cell lymphomas, 3 T-cell lymphomas and 2 cases of classical Hodgkin lymphoma seen in a total of 8 patients (26%). Clonal relationships between FL and classical Hodgkin lymphomas, and FL and mantle cell lymphoma (MCL) occurring in the same patient have been reported, so there could still be a clonal relationship between FLBUS and some of these other types of lymphoma.[28-31] While the cases of DLBCL are much more likely to be related to a pre-existing FL or FLBUS, the FISH findings in this study suggested that most did not represent transformation of the FLBUS, most of which will have *BCL2* translocations.[4,5] Others have reported presumptive clonal identity between the FLBUS and the associated higher grade B-cell lymphomas based on PCR product size.[6] Definitive comparisons would require sequencing of the PCR products. Comparisons are also complicated by the very small amount of FLBUS in some cases and the observation that even reactive germinal centers can have oligoclonal or monoclonal populations. Whether the extent of involvement of FLBUS correlates with synchronous or metachronous FL or DLBCL has not yet been established. This is important since if, for example, more extensive involvement meant it was more likely that the patient would have an overt FL/DLBCL, this could affect the nature of staging and follow-up guidelines, or potentially even therapy. Montes-Moreno *et al.* found that the amount of "intrafollicular neoplasia" was associated with a related FL or DLBCL,[4] but the larger study of Jegalian *et al.*, who used a simpler scoring system, found no significant association.[5] In this latter study, 6 of the 7 cases with an associated FL fell into the category with the least involvement, compared with only 6 of 20 cases with no associated lymphoma.[5] Our study examined in even greater detail the absolute number of involved follicles, their proportion, and the degree of involvement within individual follicles, and added the parameter of density of abnormal follicles. But we found that none of these parameters could predict the presence of an associated DLBCL, FL or FL/DLBCL. As in the other studies, it is important to remember that these data are still based on a limited number of patients with a relatively short follow up.

Typically, identification of FLBUS is based on immunohistochemical staining of tissue sections. However, more and more patients are now being evaluated using needle core biopsies or even fine needle aspiration leading to greater reliance on FCS. Currently the interpretation of finding CD10+ light chain restricted B cells or CD10+BCL2+ B cells is that it reflects a B-cell lymphoma of germinal center origin with the major exception being the rare cases of clonal follicular hyperplasias which are most typically seen in children and young adults.[32] The proportion of FLBUS that will be identified by FCS which would then require additional disclaimers when interpreting 'positive' FCS, is completely unknown. Whereas the focality of FLBUS might suggest a low proportion of cases being detected, the ability of FCS to focus on very specific lym-

Table 5. Clinicopathological features of patients with FLBUS discovered in retrospective biopsies.

Case ID	Gender	Age (yr)[a]	FLBUS biopsy site	Reason for procedure/other pathology at time of FLBUS	Biopsy site of subsequent FL	Interval from FLBUS to FL (mo)	Stage of FL[b]	Rx for FL	FU after FL (mo)	Status at FU
LB1	M	82	"Bronchial" LN	Metastatic small cell carcinoma	Peri/intraparotid LN	46	NA	None	42	A with presumptive carcinoma metastasis in brain
LB2	M	58	Submandibular LN	Part of radical neck operation for metastatic carcinoma	Right inguinal LN	108	III	R-CHOP and maintenance R	36	A with metastatic squamous cell carcinoma
LB3	F	62	Left inguinal LN	Adenopathy	Level 4R LN	1	IIIA[c]	R-CHOP and maintenance R	19	A without D
LB4	M	71	Lipoma of cord LN	Hernia repair	Left supraclavicular LN	7	III[d]	Hyper-CVAD	47	A without D

NA: not available, FL: follicular lymphoma, R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone, A without D – alive without disease. In one additional 69-year old female, FLBUS was discovered in pelvic lymph nodes from a hysterectomy specimen for endometrial adenocarcinoma 63 months prior to a grade 1-2 follicular lymphoma. This patient, however, had a clinical history of a follicular lymphoma from approximately one year prior to the hysterectomy specimen. Neither a report nor the material from the original reported follicular lymphoma was available for review and no additional clinical information was available. [a]Age at time of biopsy showing FLBUS [b]Staging did not include documented bone marrow examinations except for LB4. [c]Diffuse large B-cell lymphoma was also diagnosed at this time in a level 7 lymph node biopsy. [d]B - l y m - phoblastic leukemia/lymphoma with MYC, BCL2 and IGH rearrangements was also diagnosed at this time in a mandibular mass biopsy. Bone marrow was negative.

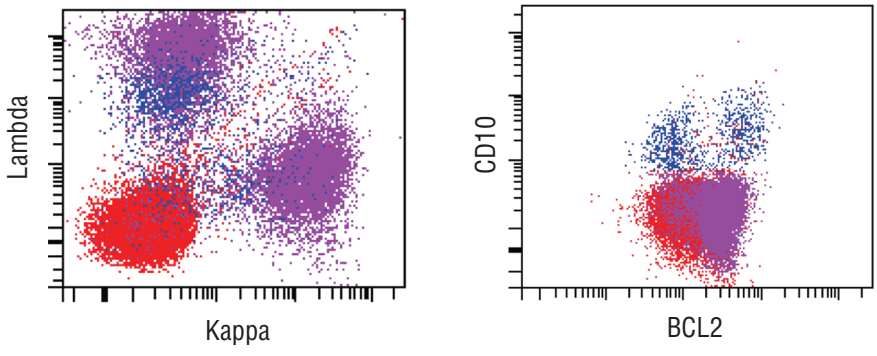

Figure 5. Flow cytometric detection of FLBUS (Case 11). (A) The CD10$^+$ B cells (blue) showed a lambda predominance whereas the CD10$^-$ B cells (purple) were polytypic. (B) The CD10$^+$ B-cells showed both a BCL2$^+$ population (shifted to the right of the other cells present) and a population that is BCL2 negative based on the criteria of Cook, *et al.*[8] This is consistent with the presence of both neoplastic (λ^+, CD10$^+$, BCL2$^+$ B-cells) and reactive (polytypic and probably surface immunoglobulin negative, CD10$^+$, BCL2$^-$ B$^-$ cells) GC cells.

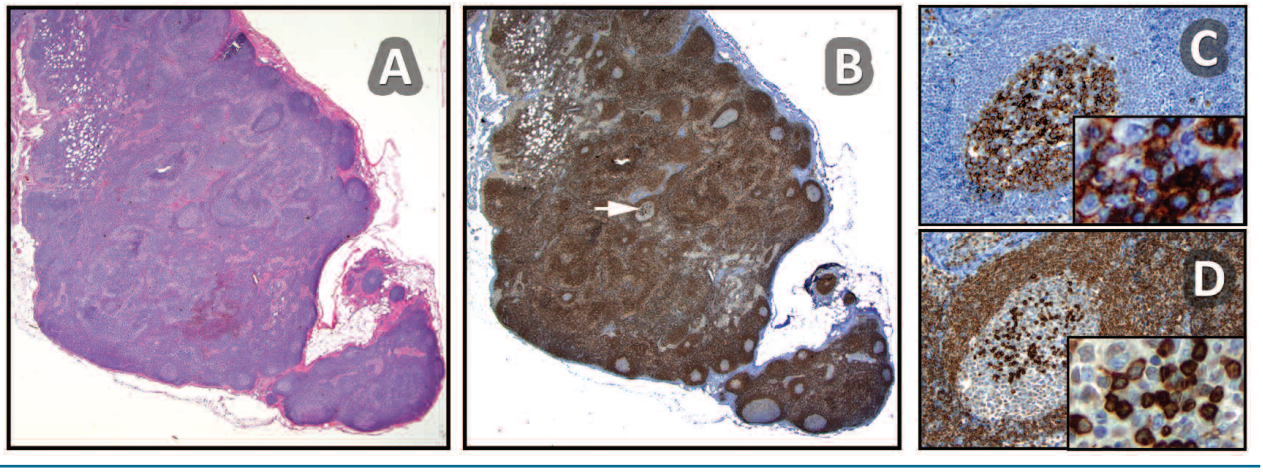

Figure 6. FLBUS detected in retrospect in lymph node from a left neck dissection for metastatic carcinoma in a 58-year old/M patient who 9 years later developed FL grade 3B. (A) The lymph node has architectural preservation and scattered follicles. (B) The BCL2 immunostain highlights a rare GC with intensely positive cells (arrowhead). (C) The antibody to CD10 marks the GC and includes some more intensely staining cells with a distribution that appears similar to the (D) distribution of the intensely BCL2$^+$ cells which represent the FLBUS. Original magnification ×40 (A-B) and ×400 (C-D).

phoid populations, even if very small, suggests the possibility of a higher yield. FCS detected CD10+ light chain restricted and/or BCL2+ B cells in approximately half of our cases. While the overall proportion of these cells was relatively low, they often made up a high proportion of the CD10+ B cells present. As might be expected, these cases were found to have a significantly higher proportion of BCL2+ follicles. A CD10+ BCL2+ B-cell population was detected in all tested cases with CD10+ light chain restricted cells and also in an additional biopsy that had a CD10+ but surface immunoglobulin negative population. These results indicate that detection of CD10+ BCL2+ and/or light chain restricted cells in FCS should now be interpreted with even greater caution, since in addition to all the other necessary disclaimers, the possibility of FLBUS, currently considered to be very different from even a low-grade FL, must be investigated. Conversely, approximately half of FLBUS were missed in the FCS, not all of which had BCL2 studies, and sampling issues could be a possible explanation.

Finally, other than a recently published abstract that reported *in situ* FL preceding overt FL in 6 of 8 cases (with the other 2 cases demonstrating undiagnosed overt FL),[33] and unpublished data mentioned in a study of MCL that describe *in situ* FL preceding overt FL in only 16% of cases,[34] it is unknown how often overt FL is preceded by FLBUS. Our findings support the view that FLBUS is a precursor of most FL, although this does not mean that focal follicular involvement by an overt FL might not mimic FLBUS in some cases. It is of interest that the only case in which we did not find FLBUS preceding the FL was one that was subsequently excluded from this study because it actually represented a primary cutaneous follicle center lymphoma, i.e. an entity considered distinct from other FL in the 2008 WHO classification. This is analogous to the situation with chronic lymphocytic leukemia (CLL)/monoclonal B-cell lymphocytosis (MBL) and possibly MCL/MCLBUS. Chronic lymphocytic leukemia-type MBL has a rate of progression to CLL of only 1-2%/year.[35-38] However, almost all CLL are preceded by MBL.[7] The situation may be similar with MCLBUS, although the proportion of MCL with preceding *in situ* MCL or small foci of cyclin D1+ lymphoid cells is controversial.[34,39,40]

This multiparameter study, therefore, demonstrates that FLBUS has a low rate of progression to overt lymphomas, at least over several years following diagnosis, but that in approximately half the cases it is associated with a synchronous or metachronous overt FL, DLBCL or other type of malignant lymphoma. In approximately half the cases, FCS will demonstrate a population of CD10+ BCL2+ often light chain restricted B cells that are indistinguishable from what would be seen in an overt FL or DLBCL. This means that caution must be exercised in using these studies to make the diagnosis of lymphoma, particularly when only dealing with cytological preparations. Unfortunately, routine histologic/immunohistological evaluations do not predict which cases are associated with overt FL/DLBCL. The critical factors that lead to the development of overt lymphomas in patients with FLBUS, and practical ways of detecting them in the face of the focal nature of the abnormal follicles, still have to be established.

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